Effectiveness of 2-D Electrophoresis over 1-D Electrophoresis Followed by Western Blotting for Evaluating Strategies Used to Generate Host Cell Protein Antibodies

Tech Note

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Introduction

Specifications for the highest degree of purity for manufactured industrial biopharmaceuticals require final products to be essentially free of host cell protein (HCP) contaminants. Host cell proteins may be left behind during the purification process from the expression hosts, such as *E. coli*, insect, or mammalian cells. If impure products are administered to patients, contaminants can result in adverse toxic or immunological reactions. To investigate the presence of residual contamination of the final biopharmaceutical product, polyclonal antibodies with maximum coverage against naïve HCP lysates are developed, providing a valuable tool for further assay development (for example, HCP ELISAs) to determine product purity (Wang et al. 2009, Champion et al. 2005).

There are several critical parameters in developing a robust antibody-based HCP detection system: the immunization strategy, the immunoassay detection methodology, and data evaluation. Several immunization strategies can be employed for the development of anti-HCP antibodies, which can be used independently or in combination to maximize immuno-coverage against a range of host cell proteins (Eaton 1995, Thalhamer and Freund 1984, Schwertner and Kirchner 2010). These strategies include fractionation of HCP extracts and immunization of host animals using each fraction separately, or using a cascade immunization (successive immunogen depletion) that uses multiple antigen boosts for maturation of a broad immune response. Note that the use of multiple host species is another protocol that can improve maximum coverage. Along with immunization strategy, a stringent evaluation method that is reliable and that enables correct determination of the optimal immunization strategies is required.

Among various evaluation methods, gel-based separation of host cell protein mixtures followed by western blotting with anti-HCP antibodies is attractive since it offers visual confirmation of immunodetected proteins. For anti-HCP antibody evaluations, 1-D electrophoresis (1-DE) by SDS-PAGE is often used in conjunction with western blotting (Tscheliessnig et al. 2013). However, the accuracy of this evaluation remains questionable because this method does not effectively resolve highly complex protein mixtures such as HCP extracts. By contrast, 2-D electrophoresis (2-DE) which separates proteins orthogonally by isoelectric point (pI) and molecular weight is an excellent alternative because it is able to resolve complex protein mixtures.

Here, we tested the feasibility of using 1-D or 2-D electrophoresis followed by western blotting for evaluating immunocoverage of novel anti-HCP antibody reagents developed against *E. coli* proteins.

Materials and Methods

Materials

ReadyPrep™ 2-D cleanup kit, ReadyPrep rehydration/sample buffer, ReadyStrip™ IPG strips, Criterion™ TGX Any kD™ gels, Trans-Blot® Turbo™ PVDF transfer packs, Oriole™ fluorescent gel stain and SYPRO Ruby blot stain were from Bio-Rad Laboratories. Blocking buffer, secondary HRP-conjugated goat anti-rabbit IgG antibody and chemiluminescent substrate were from Rockland Immunochemicals Inc. (Gilbertsville, PA). Centricon ultrafiltration cartridges were obtained from EMD Millipore (Billerica, MA).

Sample Collection and Processing

E. coli HCPs were prepared from BL21 (DE) Lys S cells, mock-transfected with empty pET28 vector, and mock-induced overnight at 25°C using 0.4 mM IPTG. Cells were lysed by sonication in PBS supplemented with protease inhibitors, treated with DNase, clarified by centrifugation, and filtered using 0.2 mm filters. *E. coli* HCPs thus generated were injected into New Zealand white rabbits either directly or after fractionation. All proteins used for immunization were verified to be low in endotoxin prior to injection.

Bleeds were collected at various time points and booster injection of the HCPs was given depending on the specific immune response of each animal. Generated antibodies were used for western blotting directly as antisera fraction without affinity purification.

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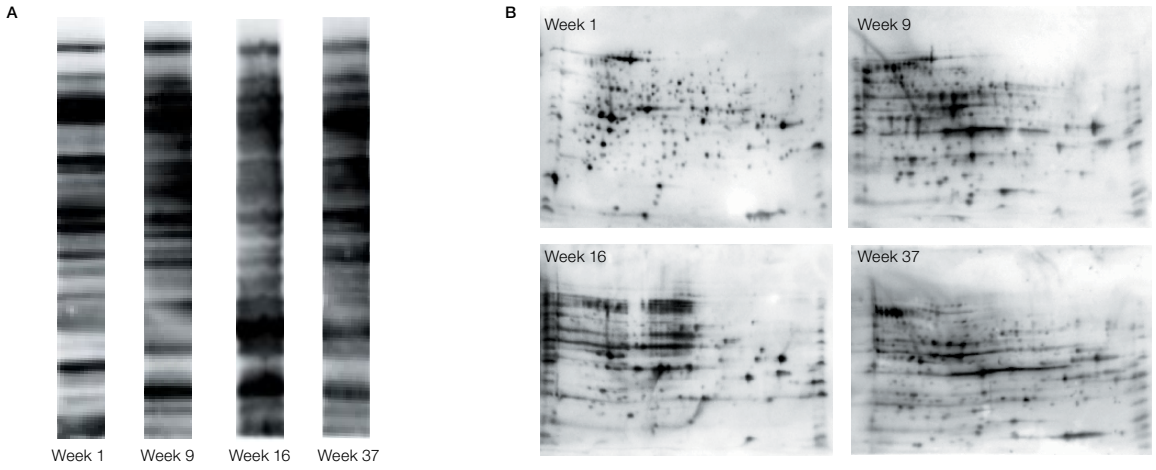

Fig. 1. Visualization of rabbit immune response maturation against *E. coli* HCP mixtures by western blotting after 1-DE versus 2-DE. A, *E. coli* HCPs were separated by 1-DE and probed using anti-HCP antibodies generated at 1, 9, 16, and 37 weeks after rabbit immunization; **B**, *E. coli* HCPs were separated by 2-DE and probed using same anti-HCP antibodies as above generated at 1, 9, 16, and 37 weeks, respectively.

Fractionation of *E. coli* HCPs was done using 100 kDa MWCO spin filters to generate a retentate (fraction 1) and filtrate (fraction 2); endotoxins were removed using Miltenyi endotoxin removal beads (Miltenyi Biotech, Auburn, CA). Each of these samples (fraction 1 and fraction 2) and unfractionated/total *E. coli* protein was separately injected into different rabbits as described above.Antibodies from total or fractionated *E. coli* HCPs were pooled from multiple animals at various weeks to achieve maximal response in western blots.

Electrophoresis and Western Blotting

For 2-DE of *E. coli* HCPs, protein samples were processed by 2-D cleanup kit and redissolved in ReadyPrep rehydration/sample buffer supplemented with DTT and ampholytes. First dimension isoelectric focusing (IEF) was done on pH 5-8 ReadyStrip IPG strips on the PROTEAN® i12™ IEF system. SDS-PAGE (as a second dimension, post IEF separation or as a stand-alone 1-DE analysis) was done using Criterion TGX Any kD gels after 1-DE or 2-DE. Proteins were transferred using the Trans-Blot Turbo system and total protein on blot was detected using Coomassie staining (1-DE) or SYPRO Ruby blot stain (2-DE). Proteins were subsequently probed with anti-HCP antibody preparations and evaluated by western blotting. For western blotting, membranes were blocked with blocking buffer and probed with anti-HCP antibody preparations. Goat anti-rabbit IgG conjugated with HRP was used as a secondary antibody. Chemiluminescent substrate was applied and blots were imaged using a ChemiDoc™ MP imaging system with spot analysis performed using PDQuest™ 2-D analysis software.

Results and Discussion

The goal of this study was to analyze the efficacy of 1-DE vs. 2-DE followed by western blotting for evaluating immunocoverage of anti-HCP antibody preparations. We used two different antibody preparations raised against *E. coli* proteins: (i) antisera collected at regular intervals over a 37 week period and (ii) antisera generated from fractionated *E. coli* extracts. Each of these antibody preparations was used to test the two anti-HCP antibody evaluation methods: 1-DE and western blotting versus 2-DE and western blotting.

First, we analyzed antibody preparations generated over a 37 week period from rabbits immunized with total *E. coli* proteins. Our interest was to determine the time point at which broadest immunocoverage against *E. coli* proteins was observed. Shown here are results of antisera bleeds collected at the following time points after immunization: 1 week, 9 weeks, 16 weeks, and 37 weeks; collections were made from four to six different animals at each time point and pooled. The pooled antisera at each time point were then evaluated for immunocoverage against *E. coli* proteins via 1-DE and western blotting (Figure 1A) versus 2-DE and western blotting (Figure 1B).

Data from 1-DE and western blotting show relatively low level of immuncoverage detectable at 1 week after immunization. Although subsequent time points (9, 16, and 37 weeks) demonstrate qualitatively higher immuno-coverage, significant relative differences between these time points are indistinguishable by 1-DE and western blotting. By contrast, progressive improvement in immunocoverage from 9 weeks to 37 weeks was clearly discernible by 2-DE and western blotting. The best results, measured in terms of number of spots and spot distribution across pI and molecular weight, were seen at 37 weeks after immunization. Thus, the higher resolution offered by 2-DE enabled a more accurate determination of the optimal time points for collecting anti-HCP antibodies against a wide range of *E. coli* proteins.

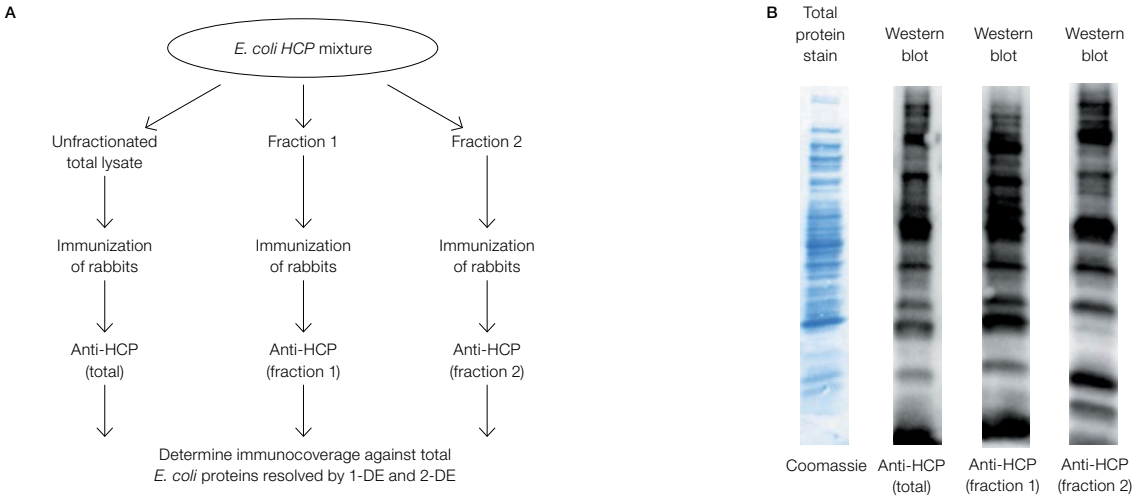

Fig. 2. Strategy for generation of anti-HCP antibodies to total vs. fractionated *E. coli* HCP mixtures and evaluation of antibodies by 1-DE and western blotting. A, Total (unfractionated) *E. coli* HCPs were directly injected into rabbits for immunization or processed for fractionation as indicated prior to rabbit immunization. Subsequently, raised antisera from each immunization were evaluated for immunocoverage against total (unfractionated) *E. coli* HCPs; **B**, Results from 1-DE total protein staining and western blotting evaluation for each of the generated antisera.

Next, we sought to determine the potential effect of fractionation of *E. coli* proteins on immunocoverage of antibodies against total *E. coli* proteins. For this determination, we again compared the efficacy of 1-DE and western blotting versus 2-DE and western blotting. To fractionate *E. coli* proteins, the total *E. coli* protein lysate (same as used previously) was processed for centrifugal filtration through a 100,000 Da MWCO filter, which resulted in two fractions: retentate (fraction 1) and filtrate (fraction 2). Total protein lysate (unfractionated), fraction 1, and fraction 2 were each injected into four to six rabbits in parallel for immunization (Figure 2A). The antisera generated using each sample [anti-HCP (total), anti-HCP (fraction 1) and anti-HCP (fraction 2)], were used to detect the total *E. coli* proteins resolved by 1-DE or 2-DE (Figures 2B and 3) by western blotting.

As shown in figure 2B, all three antisera were indistinguishable in terms of their immunocoverage by 1-DE and western blotting. However, 2-DE and western blotting demonstrated that there are potential benefits of *E. coli* protein fractionation prior to immunization (Figure 3). Compared to anti-HCP (total) antibodies, which displayed a slight bias toward higher molecular weight proteins and more acidic proteins, anti-HCP (fraction 1) and anti-HCP (fraction 2) antibodies displayed less of a pI and molecular weight bias (Figure 3). Thus, to maximize immunocoverage against the broadest range of complex HCP mixtures (for example, *E. coli* as in this study), fractionation of HCP lysates and independent immunization of each fraction is suggested. Antisera collected by such strategies could then be used either independently or after their combination to monitor the range of HCPs across pI and molecular weight.

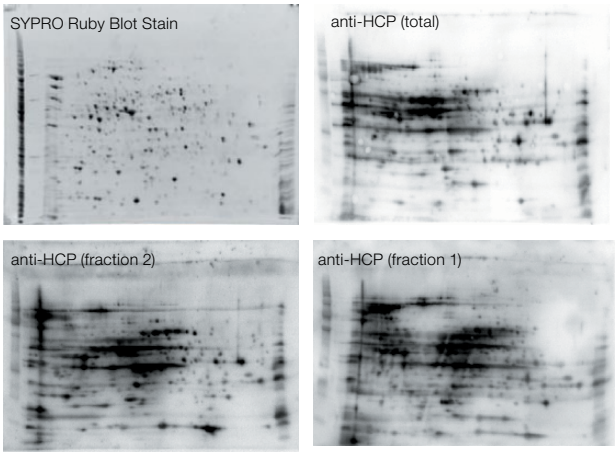

Fig. 3. 2-DE and western blotting evaluation of anti-HCP antibodies generated by immunization of rabbits with total (unfractionated) vs. fractionated *E. coli* HCP mixtures. Results from 2-DE total protein staining (on transferred proteins; SYPRO Ruby blot stain) and western blotting evaluation for each of the generated antisera [anti-HCP (total), anti-HCP (fraction 1), and anti-HCP (fraction 2)] are demonstrated.

Conclusions

During development of anti-HCP antibodies, it is critical to evaluate the effect of different strategies employed for antibody generation on the range of detectable HCPs. Both 1-DE and 2-DE followed by western blotting are commonly used for these evaluations. Here, we determined the efficacy of each of these methods for evaluating strategies used to develop anti-HCP (*E. coli*) antibodies. We demonstrate that owing to the complexity of HCP mixtures, 1-DE does

not offer adequate resolution for distinguishing various immunodetected species by subsequent western blotting. Consequently, 1-DE and western blotting was ineffective for evaluating immunization/antibody development strategies and determining the immunocoverage of anti-HCP antibodies thus generated. By contrast, evaluation using 2-DE analysis followed by western blotting demonstrated greater resolving power that was critical for effective monitoring of the immune response, and for the determination of a better final anti-HCP antibody reagent. Therefore, we recommend 2-DE over 1-DE for effective resolution of HCP mixtures, followed by western blotting for evaluating anti-HCP antibodies during and after their development.

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